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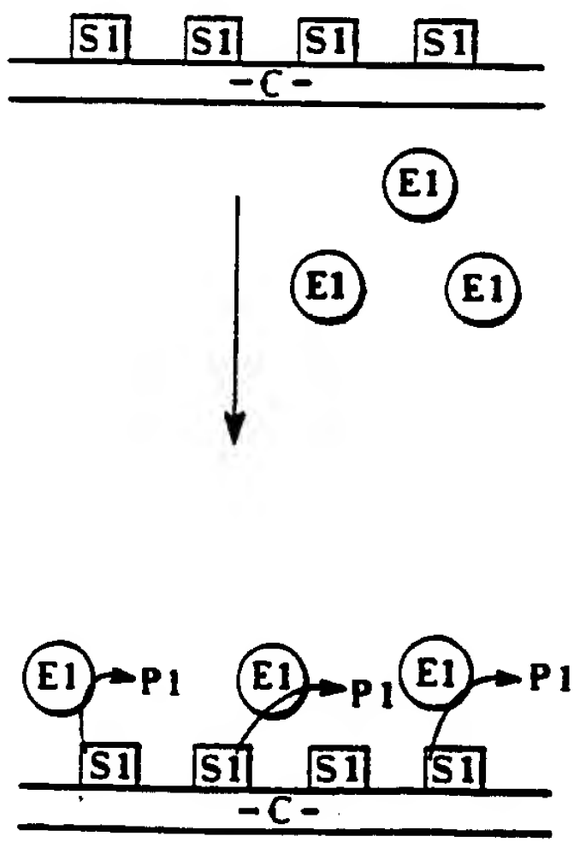
(54) Title: ANALYTICAL METHODS



(57) Abstract

A method for determining one member of an enzyme-substrate pair (the analyte), comprises bringing the members of the pair into contact so as to form, directly or indirectly, an absorbing reaction product at or in the vicinity of the surface of an optical waveguide biosensor. The biosensor is preferably a resonant optical biosensor based on the principle of frustrated total reflection.

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Title : Analytical Methods

This invention relates to methods for the qualitative or quantitative determination of biomolecules in solution, in particular to methods for the determination of enzymes and their substrates in samples of biological origin.

Many devices for the automatic determination of biochemical analytes in solution have been proposed in recent years. Typically, such devices (biosensors) include a sensitised coating layer which is located in the evanescent region of a resonant field. Detection of the analyte typically utilizes optical techniques such as, for example, surface plasmon resonance (SPR), and is based on changes in the thickness and/or refractive index of the coating layer resulting from interaction of that layer with the analyte. This causes a change, eg in the angular position of the resonance.

Other optical biosensors include a waveguide in which a beam of light is propagated. The optical characteristics of the device are influenced by changes occurring at the surface of the waveguide. One form of optical biosensor is based on frustrated total reflection. The principles of frustrated total reflection (FTR) are well-known; the technique is described, for example, by Bosacchi and Oehrle [Applied Optics (1982), 21, 2167-2173]. An FTR device for use in immunoassay is disclosed in US Patent 4,857,273 and comprises a cavity layer bounded on one side by the sample under investigation and on the other side by a spacer layer which in turn is mounted on a substrate. The substrate-spacer layer interface is irradiated with monochromatic radiation such that total reflection occurs, the associated evanescent field penetrating through the spacer layer. If the thickness of the spacer layer is correct and the incident parallel wave vector matches one of the resonant mode propagation constants, the total reflection is frustrated and radiation is coupled into the cavity layer. The cavity layer must be composed of material

which has a higher refractive ind x than the spacer layer and which is transparent at the wavelength of the incident radiation.

More recently, FTR biosensors have been described [see, for example, PCT Patent Application WO 90/06503] in which the cavity layer is a thin film of relatively high refractive index material, typically an inorganic oxide.

In all biosensors, it is necessary that the sensitised coating layer comprise a layer of immobilised chemical or biochemical species. Methods have been disclosed [see PCT Patent Application No WO 90/11510] for the determination of one member of an enzyme-substrate pair which comprise immobilising the other member of that pair on the surface of an SPR biosensor and monitoring the effect of deposition of the reaction product on the surface of the device. Obviously, to have an effect on the characteristics of the device, the reaction product must be insoluble and this places great constraints on the range of enzymes and substrates which may be determined by this method. Also, the deposition of the insoluble product on the surface means that the device is generally not re-usable.

We have now devised methods of determining enzymes and their substrates using a biosensor which overcome or substantially mitigate these disadvantages.

According to the invention, there is provided a method of determining one member of an enzyme-substrate pair (the analyte), which comprises bringing the members of the pair into contact so as to form, directly or indirectly, an absorbing reaction product at or in the vicinity of the surface of an optical waveguide biosensor.

The method of the invention is advantageous in that enzymes and substrates which produce soluble, absorbing reaction

products are more widely available than those which produce insoluble products. This means that the number of enzymes and substrates which can be directly assayed is significantly greater. In many cases appropriate substrates can be easily synthesised de novo, which is not the case for substrates which result in insoluble products. Also, if it is the enzyme which is immobilised on the sensor surface, and if the enzymes maintain their activity, the sensor can be re-used for several samples. Again, this is not the case for insoluble reaction products.

Any convenient parameter of the emitted radiation may be monitored. Obviously, the absorbing nature of the reaction product will have an effect on the intensity of the radiation coupled out of the waveguide. The product may also be fluorescent or luminescent and it may be the fluorescence or luminescence which is monitored.

One specific embodiment of the method according to the invention comprises the steps of

a) contacting a sample, in which the analyte is to be determined, with the surface of an optical waveguide biosensor on which the other member of the enzyme-substrate pair is immobilised, so as to form (if the analyte is present) a soluble, absorbing reaction product,

b) irradiating the biosensor with a beam of electromagnetic radiation such that propagation of light of a wavelength absorbed by the reaction product occurs within the waveguide, and

c) monitoring the radiation emitted from the biosensor.

The method of the invention may also be used to determine analytes which influence the rate of the enzyme-substrate reaction, ie enzyme inhibitors and activators. In this case, the method may comprise

a) contacting a sample, in which an inhibitor or activator of an enzyme is to be determined, with the surface of an

optical waveguide biosensor on which an enzyme or a substrate therefor is immobilised, the substrate being one which in the presence of the enzyme produces a soluble, absorbing reaction product,

b) adding to the sample the other member of the enzyme-substrate pair,

c) irradiating the biosensor with a beam of electromagnetic radiation such that propagation of light of a wavelength absorbed by the reaction product occurs within the waveguide, and

d) monitoring the radiation emitted from the biosensor.

In this method, the formation of absorbing reaction product correlates with the concentration of activator in the sample, or correlates inversely with the concentration of inhibitor.

In a variation on the method of the invention, where the analyte is the substrate which does not produce an absorbing reaction product, a substrate analogue which does do so may be added to the sample before contacting it with the sensor surface. The substrate analogue then competes with the natural substrate for binding to the immobilised enzyme.

In another variation, the invention also provides a method for determining one member of an enzyme-substrate pair (the analyte), which comprises

a) contacting a sample, in which the analyte is to be determined, with the surface of an optical waveguide biosensor on which a second enzyme is immobilised,

b) adding to the sample the other member of the enzyme-substrate pair and a substrate for the immobilised enzyme which, in the presence of a reaction product of the enzyme-substrate pair, produces a soluble, absorbing reaction product,

c) irradiating the biosensor with a beam of electromagnetic radiation such that propagation of light of a wavelength absorbed by the reaction product occurs within the waveguid ,

and

d) monitoring the radiation emitted from the biosensor.

Where the analyte is the substrate, both enzymes may be immobilised on the sensor surface. One enzyme reacts with the substrate in the sample to produce a product which in the presence of the substrate for the second enzyme reacts to form the absorbing, soluble reaction product.

The soluble reaction products must not diffuse out of the evanescent field of the optical waveguide sensor during the course of the assay, but must remain localised close to the immobilised enzyme. This is known to occur for some enzymes. For example, in the assay of alkaline phosphatase using the substrate dioxetane phosphate in the presence of a detergent-solubilised derivative of fluorescein [Schaap et al (1989) Clin Chem 35, 1863-1864], the luminescent signal produced remains localised close to the enzyme active site. For those enzymes for which this is not the case the reaction product may be kept within the evanescent field by means of a physical barrier or by capture on the sensor surface by means of an antibody. For example, antibodies may be prepared which bind the product of the enzyme reaction but not the substrate. The antibodies may be immobilised on the same chip as the enzymes.

If the reaction products are constrained to the vicinity of the surface at which they are formed, it may be possible to detect several analytes within the same sample.

Examples of some suitable enzyme-substrate pairs are the following:

	Absorbing product
ß-galactosidase	resorufin-ß-D-galactopyranoside
ß-galactosidase	methoxy-1-naphthyl-ß-D-galactopyranoside
ß-glucuronidase	resorufin-ß-D-glucuronide

<u>Fluorescent product</u>	
siayltransferases	cytidine-5-monophosphate-(3-fluoresceinyl-thioureido)-deoxy-N-acetyl-neuramic acid
ß-galactosidase	fluorescein di-(ß-D-galactopyranoside)
<u>Luminescent product</u>	
alkaline phosphatase	dioxetane phosphate in the presence of a detergent-solubilised derivative fluorescein
firefly luciferase	ATP and luciferin

Assays for some enzymes may be performed by observing a decrease in the absorbance at a particular wavelength. For example, angiotensin converting enzyme may be assayed with the substrate FA-Phe-Gly-Gly (FA is furylacryloyl) by observing a decrease in absorbance.

Enzymes for which there are no suitable substrates for direct measurement may be detected by the use of a coupling enzyme. For example, oxalate oxidase may be detected as follows:

$$(COOH)_2 + O_2 \xrightarrow[\text{oxalate oxidase}]{} 2CO_2 + H_2O_2$$

$$H_2O_2 + \text{MBTH} + \text{DMA} \xrightarrow[\text{peroxidase}]{} \text{Indamine dye} + 2H_2O$$

MBTH = 3-methyl-2-benzothiazolinone hydrazone
DMA = N,N-dimethylaniline

Alternatively, a suitable, simple chemical reaction which uses the reaction product to produce an absorbing species may be available. For example, creatine kinase or phosphocreatine may be assayed as follows:

$$\text{phosphocreatine} + \text{ADP} \xrightarrow[\text{creatinine kinase}]{} \text{creatinine} + \text{ATP}$$

$$\text{creatinine} + \text{naphthol} + \text{diacetyl} \longrightarrow \text{pink complex}$$

The methods according to the invention may be conducted with any form of optical waveguide biosensor. However, it is preferred to use a resonant biosensor based on the principle of frustrated total reflection (FTR). Such a biosensor typically comprises

a) a cavity layer of dielectric material of refractive index n_3,

b) a dielectric substrate of refractive index n_1, and

c) interposed between the cavity layer and the substrate, a dielectric spacer layer of refractive index n_2.

In use, the interface between the substrate and the spacer layer is irradiated with light such that total reflection occurs. In this context, 'light' may include not only visible light but also wavelengths above and below this range, eg in the ultra-violet and infra-red.

Resonant propagation of a guided mode in the cavity layer will occur, for a given wavelength, at a particular angle of incidence of the exciting radiation. Thus, two basic measurement approaches are possible: scanning the angle of incidence at fixed wavelength or scanning the wavelength at a fixed angle of incidence. The former approach, using monochromatic radiation, is preferred since it allows the use of a laser source, simplifying the problem of optical collimation, and avoids dispersion effects, thereby simplifying the analysis of the results.

The angular position of the resonant effect depends on various parameters of the biosensor device, such as the refractive indices and thicknesses of the various layers. In general, it is a pre-requisite that the refractive index n_3 of the cavity layer and the refractive index n_1 of the substrate should both exceed the refractive index n_2 of the spacer layer. Also, since at least one mode must exist in the cavity to achieve resonance, the cavity layer must exceed a certain minimum thickness.

The cavity layer is preferably a thin-film of dielectric material. Suitably transmissive dielectric materials for the cavity layer include zirconium dioxide, titanium dioxide, aluminium oxide and tantalum oxide.

The cavity layer may be prepared by known techniques, eg vacuum evaporation, sputtering, chemical vapour deposition or in-diffusion.

The dielectric spacer layer must also be suitably transmissive to the incident radiation and must have a lower refractive index than both the cavity layer and the substrate. The layer may, for example, comprise an evaporated or sputtered layer of magnesium fluoride. In this case an infra-red light injection laser may be used as light source. The light from such a source typically has a wavelength around 800nm. Other suitable materials include lithium fluoride and silicon dioxide. Apart from the evaporation and sputtering techniques mentioned above, the spacer layer may be deposited on the substrate by a sol-gel process, or be formed by chemical reaction with the substrate.

The refractive index of the substrate (n_1) must be greater than that (n_2) of the spacer layer but the thickness of the substrate is generally not critical to the performance of the invention.

By contrast, the thickness of the cavity layer must be so chosen that resonance occurs within an appropriate range of coupling angles. The spacer layer will typically have a thickness of the order of several hundred nanometres, say from about 200nm to 2000nm, more preferably 500 to 1500nm, eg 1000nm. The cavity layer typically has a thickness of a few tens of nanometres, say 10 to 200nm, more preferably 30 to 150nm, eg 100nm.

It is particularly preferred that the cavity layer has a

thickness of 30 to 150nm and comprises a material selected from zirconium dioxide, hafnia, silicon nitride, titanium dioxide, tantalum oxide and aluminium oxide, and the spacer layer has a thickness of 500 to 1500nm and comprises a material selected from magnesium fluoride, lithium fluoride and silicon dioxide, the choice of materials being such that the refractive index of the spacer layer is less than that of the cavity layer.

Preferred materials for the cavity layer and the spacer layer are tantalum oxide and silicon dioxide respectively.

Any convenient source of radiation may be used as the source of the incident light but it is preferable to use monochromatic radiation and the most convenient source of such radiation is a laser. The choice of laser will depend inter alia on the materials used for the various layers of which some examples have already been given, and of course on the particular absorbing reaction product.

The scanning of angle may be performed either sequentially or simultaneously ie by varying the angle of incidence of a parallel beam of light or by simultaneously irradiating over a range of angles using a fan-shaped beam of light as described (in connection with SPR) in European Patent Application No 0305109A. In the former case, a single-channel detector may be used which is mechanically scanned over a range of angles; in the latter case, in which a range of angles is irradiated simultaneously, it will generally be necessary to use a multi-channel detector having angular resolution.

At resonance, the incident light is coupled into the cavity layer by FTR, propagates a certain distance along the cavity layer, and couples back out (also by FTR). The propagation distance depends on the various device parameters but is typically of the order of 1 or 2mm.

The formation of absorbing reaction product in the course of the methods according to the invention may result in a reduction in the intensity of the reflected light. If the reaction product is fluorescent or luminescent, the increase in intensity at the fluorescent or luminescent wavelengths may be used to determine the analyte.

Some methods according to the invention will now be illustrated further with reference to the accompanying Figures.

Figure 1 shows schematically an assay method for the determination of an enzyme E1. A substrate S1 for the enzyme is immobilised on the surface of the cavity layer C of an FTR biosensor. The substrate S1 may be immobilised directly on the layer C or may be immobilised in a layer of gel. When a sample containing the enzyme E1 is brought into contact with the biosensor surface C, a detectable (absorbing) product P1 is produced.

Figure 2 shows a corresponding method for the determination of a substrate S. In this case, it is the enzyme E which is immobilised on the sensor surface C.

In the scheme shown in Figure 3 for the determination of an enzyme E1, a coupling enzyme E2 is immobilised on the surface C. To the sample containing E1 is added a substrate S1 for that enzyme and a substrate S2 for the coupling enzyme E2 which in the presence of the product P1 (produced from S1 by the action of E1) produces a detectable (absorbing) product P2.

Figure 4 shows a scheme in which the substrate S1 is the analyte and two enzymes E1 and E2 are immobilised on the sensor C. A coupling substrate S2 is added to the sample containing S1. The analyte substrate S1 is converted by enzyme E1 to a product P1 which r acts, in the presence of

enzyme E2, with the coupling substrate S2 to produce a detectable (absorbing product) P2.

Figure 5 shows a variation on the scheme shown in Figure 2 in which the reaction product P is captured and retained at the surface C of the sensor by immobilised specific antibodies.

Claims

1. A method of determining one member of an enzyme-substrate pair (the analyte), which comprises bringing the members of the pair into contact so as to form, directly or indirectly, an absorbing reaction product at or in the vicinity of the surface of an optical waveguide biosensor.

2. A method as claimed in Claim 1, which comprises the steps of

a) contacting a sample, in which the analyte is to be determined, with the surface of an optical waveguide biosensor on which the other member of the enzyme-substrate pair is immobilised, so as to form (if the analyte is present) a soluble, absorbing reaction product,

b) irradiating the biosensor with a beam of electromagnetic radiation such that propagation of light of a wavelength absorbed by the reaction product occurs within the waveguide, and

c) monitoring the radiation emitted from the biosensor.

3. A method as claimed in Claim 1, for the determination of an analyte which influences the rate of an enzyme-substrate reaction, which method comprises

a) contacting a sample containing the analyte with the surface of an optical waveguide biosensor on which an enzyme or a substrate therefor is immobilised, the substrate being one which in the presence of the enzyme produces a soluble, absorbing reaction product,

b) adding to the sample the other member of the enzyme-substrate pair,

c) irradiating the biosensor with a beam of electromagnetic radiation such that propagation of light of a wavelength absorbed by the reaction product occurs within the waveguide, and

d) monitoring the radiation emitted from the biosensor.

4. A method as claimed in Claim 1, wherein the analyte is the substrate which does not produce an absorbing reaction product, and the method includes the further step of adding a substrate analogue which does do so to the sample before contacting it with the sensor surface.

5. A method as claimed in Claim 1, which comprises
a) contacting a sample, in which the analyte is to be determined, with the surface of an optical waveguide biosensor on which a second enzyme is immobilised,
b) adding to the sample the other member of the enzyme-substrate pair and a substrate for the immobilised enzyme which, in the presence of a reaction product of the enzyme-substrate pair, produces a soluble, absorbing reaction product,
c) irradiating the biosensor with a beam of electromagnetic radiation such that propagation of light of a wavelength absorbed by the reaction product occurs within the waveguide, and
d) monitoring the radiation emitted from the biosensor.

6. A method as claimed in Claim 5, wherein both enzymes are immobilised on the sensor surface.

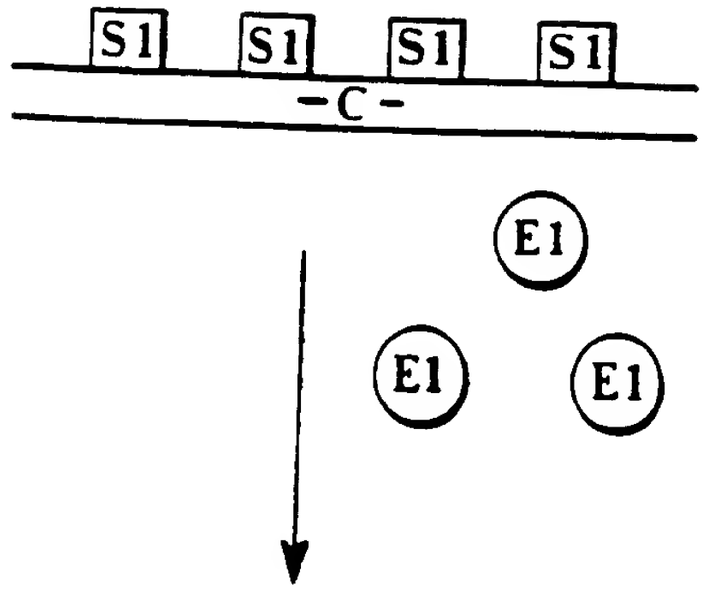
7. A method as claimed in any one of the preceding claims, wherein reaction product is constrained in the vicinity of the sensor surface by means of a physical barrier or by capture on the sensor surface by means of an antibody.

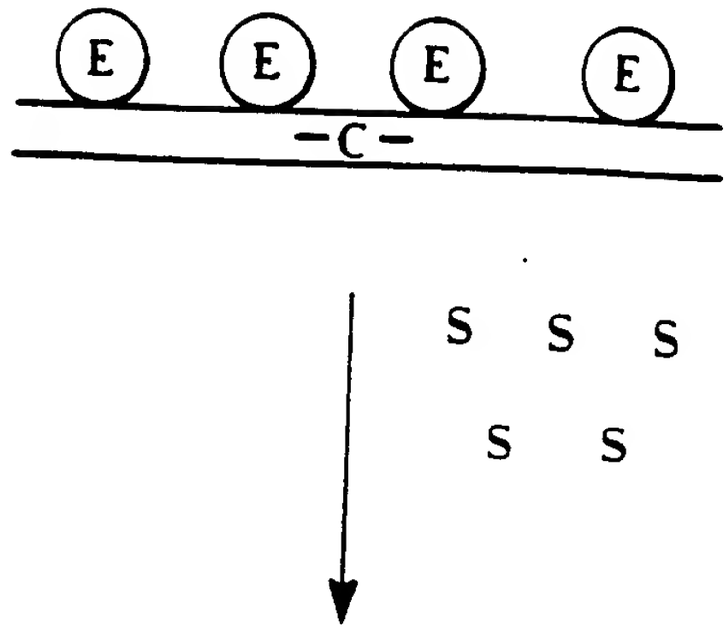
8. A method as claimed in any one of the preceding claims, wherein the optical waveguide biosensor is a resonant biosensor based on the principle of frustrated total reflection comprising
a) a cavity layer of dielectric material of refractive index n_3,
b) a dielectric substrate of refractive index n_1, and
c) interposed between the cavity layer and th substrate, a

dielectric spacer layer of refractive index n_2.

9. A method as claimed in Claim 8, wherein the cavity layer is a thin-film of dielectric material.

10. A method as claimed in Claim 8, wherein the cavity layer has a thickness of 30 to 150nm and comprises a material selected from zirconium dioxide, hafnia, silicon nitride, titanium dioxide, tantalum oxide and aluminium oxide, and the spacer layer has a thickness of 500 to 1500nm and comprises a material selected from magnesium fluoride, lithium fluoride and silicon dioxide, the choice of materials being such that the refractive index of the spacer layer is less than that of the cavity layer.





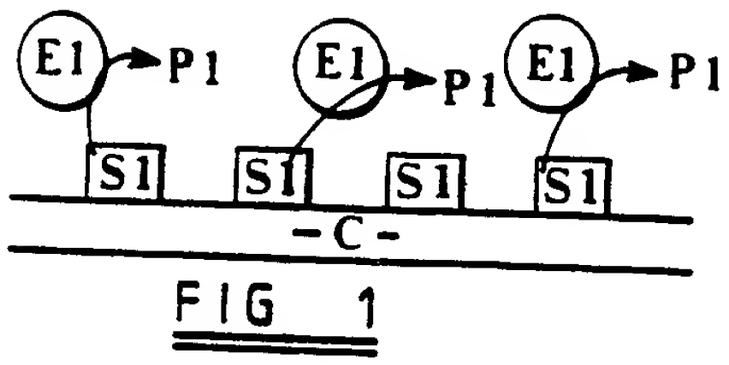


FIG 1

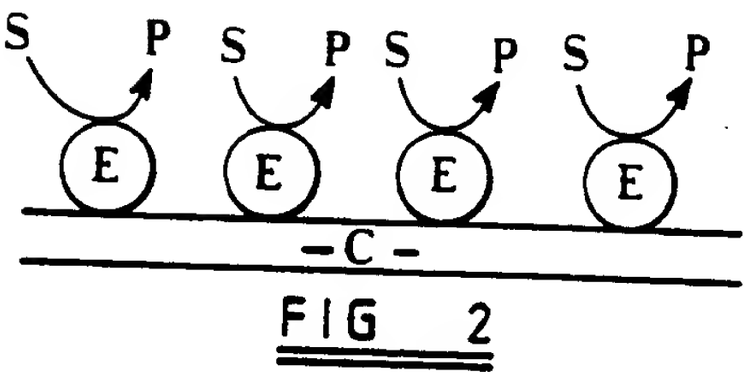


FIG 2

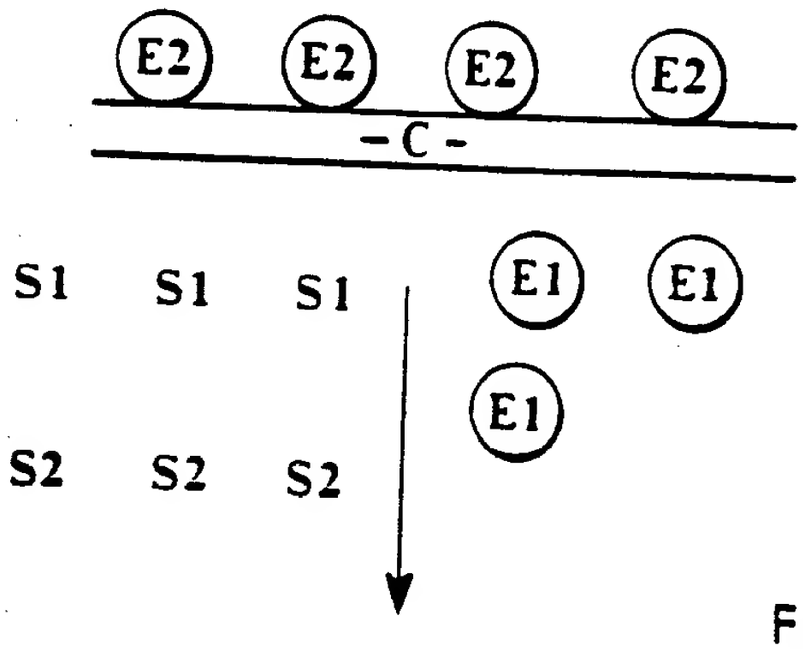
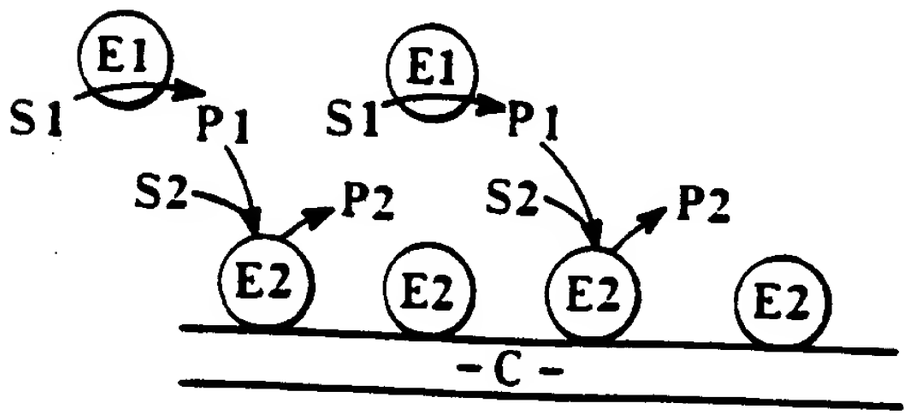


FIG 3



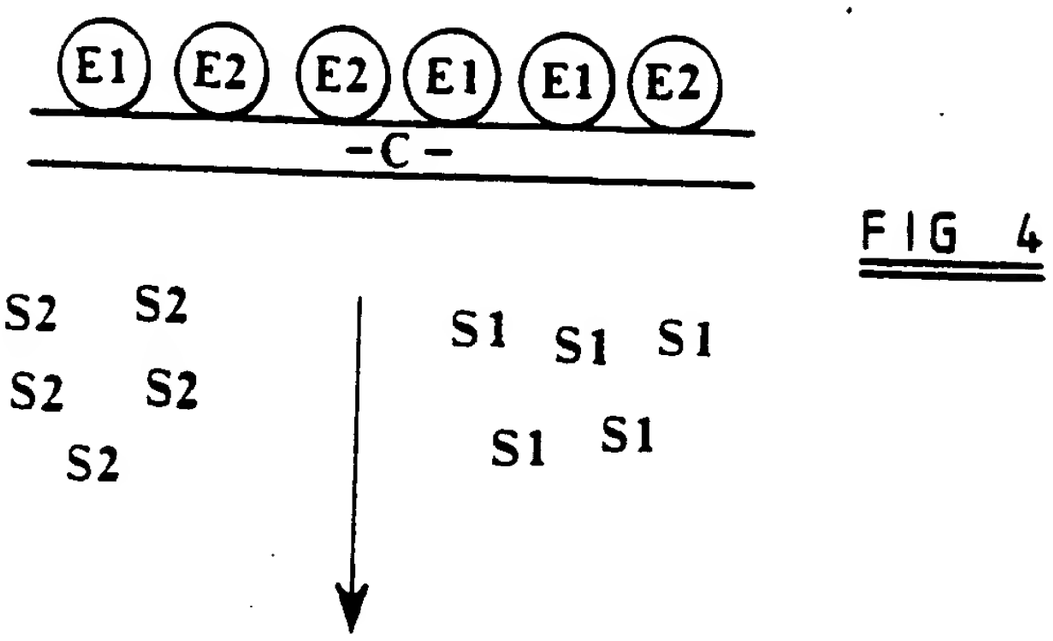

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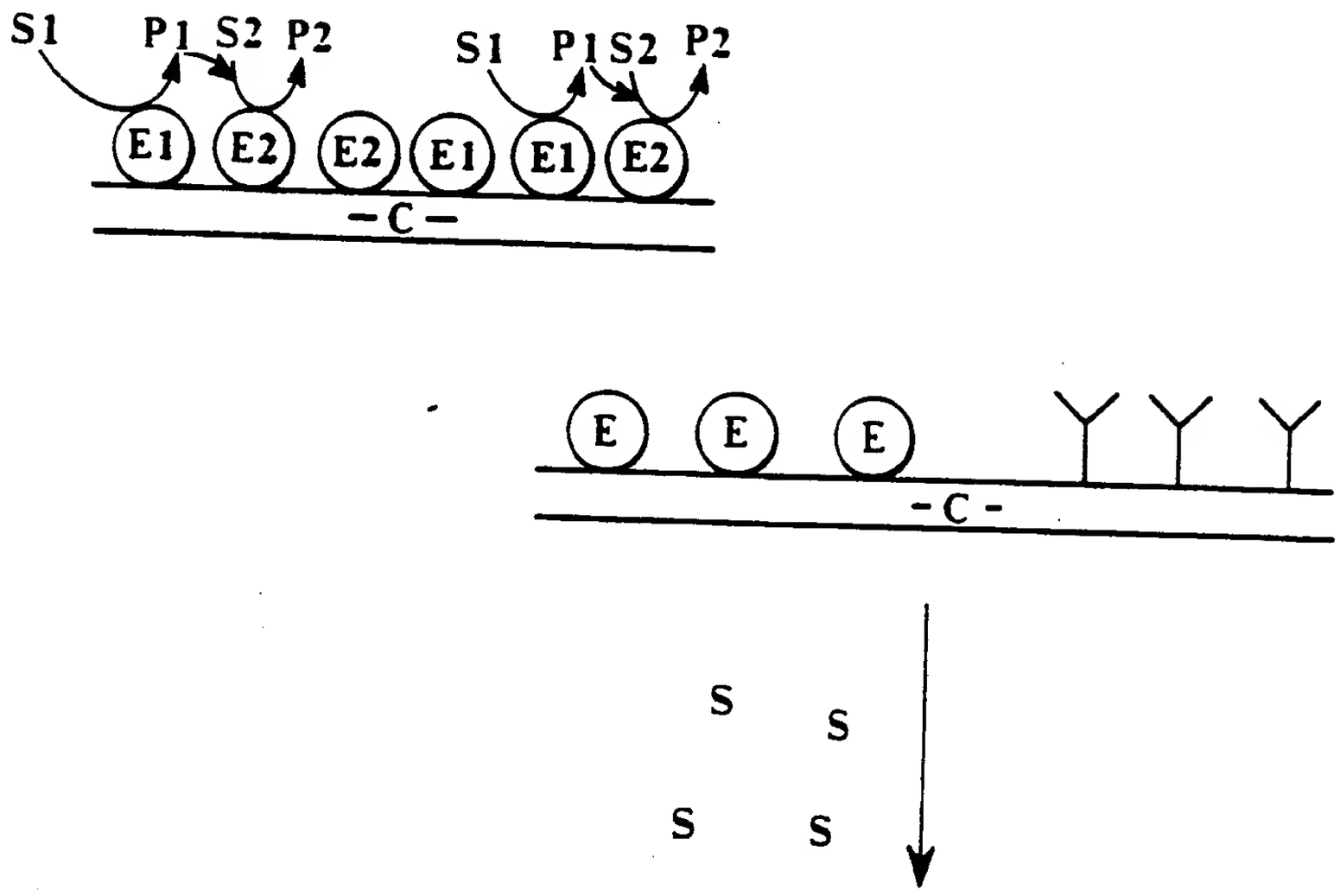
FIG 4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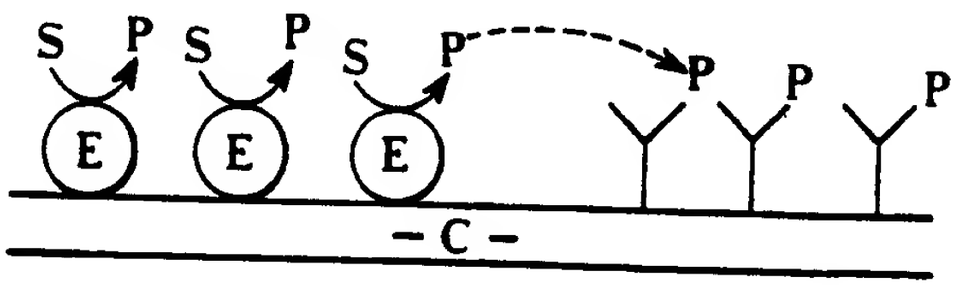
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FIG 5

INTERNATIONAL SEARCH REPORT

International Application No PCT/GB 92/00991

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all)[6]

According to International Patent Classification (IPC) or to both National Classification and IPC

Int.Cl. 5 C12Q1/00; C12M1/40; G01N33/48

II. FIELDS SEARCHED

Minimum Documentation Searched[7]

Classification System	Classification Symbols
Int.Cl. 5	C12Q ; G01N

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched[8]

III. DOCUMENTS CONSIDERED TO BE RELEVANT[9]

Category °	Citation of Document, [11] with indication, where appropriate, of the relevant passages [12]	Relevant to Claim No.[13]
A	WO,A,9 011 510 (AMERSHAM INTERNATIONAL PLC.) 4 October 1990 cited in the application see the whole document ---	1-6
A	WO,A,9 006 503 (ARES-SERONO RESEARCH & DEVELOPMENT LIMITED.) 14 June 1990 cited in the application see page 1 - page 7; figures ---	1-10

-/--

° Special categories of cited documents : [10]

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IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
04 AUGUST 1992	25.08.92
International Searching Authority	**Signature of Authorized Officer**
EUROPEAN PATENT OFFICE	HITCHEN C.E.

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category *	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No.
A	CLINICAL CHEMISTRY vol. 35, no. 9, 1989, pages 1863 - 1864; A.P.SCHAAP ET AL.: 'Chemiluminescent Substrates for Alkaline Phosphatase: Application to Ultrasensitive Enzyme-Linked Immunoassays and DNA Probes.' cited in the application see the whole document	1

ANNEX TO THE INTERNATIONAL SEARCH REPORT ON INTERNATIONAL PATENT APPLICATION NO. GB 9200991 SA 59780

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report. The members are as contained in the European Patent ffice EDP file on
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Patent document cited in search report	Publication date	Patent family member(s)		Publication date
WO-A-9011510	04-10-90	EP-A-	0464119	08-01-92
WO-A-9006503	14-06-90	AU-A-	4755690	26-06-90
		EP-A-	0455642	13-11-91